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Abbreviations:

BALF	Bronchoalveolar Lavage Fluid
DM	Dispersion Medium
ENM	Engineered Nanomaterial
IT	Intratracheal Instillation
LDH	Lactate Dehydrogenase
MWCNT	Multi-Walled Carbon Nanotube
O-MWCNT	Original MWCNT
F-MWCNT	Functionalized MWCNT
P-MWCNT	Purified MWCNT
OPA	Oropharyngeal Aspiration
PMN	Polymorphonuclear Cell
TiO_2	Titanium Dioxide
TiO_2-A	Anatase TiO_2
TiO_2-P25	Rutile/Anatase TiO_2
TiO_2-NB	TiO_2 Nanobelts

ABSTRACT

BACKGROUND: Engineered nanomaterials (ENMs) have potential benefits, but also present safety concerns for human health. Inter-laboratory studies in rodents using standardized protocols are needed for ENM toxicity assessment.

METHODS: Four labs evaluated lung responses in C57BL/6 mice to ENMs delivered by oropharyngeal aspiration (OPA). Three labs evaluated Sprague-Dawley (SD) or Fisher (F)344 rats following intratracheal instillation (IT). ENMs tested were three forms of titanium dioxide (TiO_2); anatase/rutile spheres (TiO_2-P25), anatase spheres (TiO_2-A), anatase nanobelts (TiO_2-NB), and three forms of multiwalled carbon nanotubes (MWCNT); original (O), purified (P), and carboxylic acid "functionalized" (F). Bronchoalveolar lavage fluid was collected after 1 day for differential cell counts, lactate dehydrogenase (LDH), and protein. Lungs were fixed for histopathology. Responses were also examined at 7 days (TiO_2) and 21 days (MWCNTs).

RESULTS: TiO_2-A, TiO_2-P25, and TiO_2-NB caused significant neutrophilia in mice at 1 day in 3 out of 4 labs, respectively. TiO_2-NB caused neutrophilia in rats at 1 day in 2 out of 3 labs, while TiO_2-P25 or TiO_2-A had no significant effect in any of the labs. Inflammation induced by TiO_2 in mice and rats resolved by day 7. All MWCNT types caused neutrophilia at 1 day in 3 out of 4 mouse labs and all rat labs. Three out of 4 labs observed similar histopathology to O-MWCNT or TiO_2-NB in mice.

CONCLUSIONS: ENMs produced similar patterns of neutrophilia and pathology in rats and mice. Although inter-laboratory variability was found in the degree of neutrophilia caused by the three types of TiO_2 nanoparticles, similar findings of relative potency for the three types of MWCNTs were found across all laboratories, thus providing greater confidence in these inter-laboratory comparisons.

INTRODUCTION

The nanotechnology industry is rapidly developing, resulting in the production of a variety of engineered nanomaterials (ENMs) for structural support, electronics, energy, medical imaging, and drug delivery, and other applications. While nanotechnology offers enormous potential societal benefits, concerns about the safety of ENM-containing products in regards to human and environmental health have been raised. Cumulative evidence suggests that some ENMs may exert adverse effects on the lung and other organ systems (Xia et al. 2009). These potential risks must be addressed in order to develop safe nanotechnology, but setting and implementation of exposure standards requires predictable results proven reliable by repeatability and agreement among multiple investigators. Due to the lack of standard protocols and reagents, ENM toxicity studies are difficult to compare because of inconsistencies in health outcomes and/or toxic thresholds. Much of this discordance can be attributed to the following: 1) heterogeneity of ENMs from batch to batch; 2) inherent difficulties of inter-laboratory comparisons; 3) agglomeration of particles, which often changes toxicity; 4) method and duration of dosing and dose level; and 5) method of ENM manipulation prior to testing.

In addition to dose, there are multiple factors that influence the toxicity of ENMs, including surface characteristics, charge, and shape. Size alone is a major determinant as many bulk materials that are relatively inert become toxic when produced at the nanoscale (Borm et al. 2006; Nel et al. 2006). Commonly produced and high production-volume ENMs are carbon-based (*e.g.* nanotubes, graphene, fullerenes), metal-based (*e.g.* gold, silver, quantum dots, titanium dioxide, zinc oxide), and those of a biologic nature (*e.g.* liposomes and viruses designed for gene or drug delivery) (Card et al. 2008). Determination of which ENMs will present the greatest potential threat to human health depends on relative toxicity, and on the potential for

exposure. Titanium dioxide (TiO_2) is one of the most widely used nanoscale materials to date and the conversion of bulk to nanoscale TiO_2 in consumer products (e.g. sunscreens) and industrial products (e.g. paints) is rapidly increasing (Robichaud et al. 2009). From a human health standpoint this is significant, since bulk TiO_2 nanoparticles demonstrate increased toxicity as compared to larger TiO_2 particles (Oberdorster et al. 2005). Moreover, TiO_2 nanoparticles can be manipulated into wire and belt shapes. The production of multi-walled carbon nanotubes (MWCNT) is also rapidly increasing, along with a diversity of manipulations to alter physical and chemical attributes for various applications in industry, electronics, and medicine. A number of studies have already shown that some types of MWCNT delivered to the lungs by inhalation, intratracheal instillation, or oropharyngeal aspiration cause inflammation and fibrosis (Bonner 2010).

The goal of this inter-laboratory, multi-investigator project was to determine whether independent investigators involved in NIEHS-funded Consortium studies could generate consistent data sets in rodents using a well-characterized and commonly-sourced panel of ENMs (see Xia et al. 2013) and harmonized protocols for nanoparticle dispersion, delivery to the lungs, and collection of tissues. ENMs tested by this Consortium included titanium dioxide (TiO_2) anatase/rutile nanospheres (TiO_2-P25), 100% anatase spheres (TiO_2-A) and anatase nanobelts (TiO_2-NB) as well as three different multi-walled carbon nanotubes (MWCNT), including the original material (O-MWCNT), a purified form with partial metal removal (P-MWCNT) and a carboxylic acid functionalized form (F-MWCNT). Four laboratories evaluated lung responses in C57BL/6 mice exposed to ENMs by oropharyngeal aspiration (OPA) exposure and three laboratories tested responses in Sprague Dawley (SD) or Fischer 344 (F344) rats following intratracheal instillation (IT) exposure. The results presented and discussed herein demonstrate

that a standard protocol can be used across multiple laboratories to yield similar results in the pulmonary inflammatory response. We also discuss recommendations for future directions in testing ENMs in a harmonized fashion amongst multiple laboratories that should serve to guide regulatory agencies in making decisions regarding standard setting for occupational and environmental exposures to ENMs that present potential risks to human health and the environment.

METHODS AND MATERIALS

Engineered Nanomaterials (ENMs)*:* The physical and chemical characteristics of nanoparticles used in this study are described in detail in Xia et al. 2013. Three different formulations of TiO_2 nanoparticles were rutile/anatase nanospheres (hereafter referred to as TiO_2-P25), 100% anatase nanospheres (TiO_2-A), and anatase nanobelts (TiO_2-NB). The anatase nanospheres were obtained from Dr. Pratim Biswas (Department of Energy, Environmental & Chemical Engineering, Washington University, St. Louis, MO). TiO_2 rutile/anatase P25 nanoparticles were obtained from Evonik (Essen, Germany). AnataseTiO_2 nanobelts were obtained from the laboratory of Dr. Nianqiang Wu (Mechanical and Aerospace Engineering, West Virginia University, Morgantown, WV). MWCNTs were obtained from Dr. Somenath Mitra (New Jersey Institute of Technology, Department of Chemistry and Environmental Science). An original form termed O-MWCNT from CheapTubes, Inc. (Brattleboro, VT) and two modified forms of O-MWCNT were tested. P-MWCNT was derived from O-MWCNT by acid purification to remove residual metal catalyst. F-MWCNT was derived from O-MWCNT by the addition of –COOH groups via carboxylic acid treatment to the nanotube surface.

Preparation of ENM Suspensions: Disaturated phosphatidylcholine in 100% ethanol (DSPC, Sigma-Aldrich, St. Louis, MO), rat, mouse, or bovine serum albumin (Sigma-Aldrich), and 0.9% sterile saline was used to make dispersion medium (DM) (Porter et al. 2008), in which ENMs were suspended. Spherical TiO_2 nanoparticles and MWCNT suspensions were dispersed using a cup-horn sonicator (3 mouse labs) for 1 min or probe sonicator (rat labs and 1 mouse lab) for 30 minutes using a 10 second on/off duty cycle and ice bath to disperse the particles and ensure that sample temperature did not exceed 28^{o}C. TiO_2 nanobelts were not sonicated as preliminary studies showed that this causes axial fractures. Instead, TiO_2 nanobelts were suspended in DM using gentle mechanical stirring for 60 min at room temperature. The DM alone was delivered to control animals and was also sonicated/stirred as described above.

Consortium Laboratories: Four laboratories evaluated the lung responses of C57BL/6 mice exposed to ENMs by OPA exposure and three laboratories tested responses in Sprague-Dawley or Fischer 344 rats using IT exposure. There were four mouse lab groups (ML1-ML4): East Carolina University, Michigan State University, North Carolina State University, and University of Washington. There were three rat lab groups (RL1-RL3): NIOSH, University of California Davis, and University of Rochester. Lab codes were randomly assigned and no identification is implied herein. The first round of in vivo studies involved three independent laboratories that all used SD rats that were exposed to the two spherical TiO_2-P25 or TiO_2-Ain DM. As will be described herein, none of these groups reported statistically significant changes in lung inflammatory parameters relative to DM controls. This prompted an expansion in scope of the Consortium studies to include another rat strain (F344) and another species (mouse, C57BL6). It was reasoned that this would strengthen conclusions about the relative toxicity of the selected ENMs that were evaluated.

Experimental Design: Lung tissues and bronchoalveolar lavage fluid (BALF) were collected at 1 and 7 days after exposure to TiO_2 nanoparticles and 1 and 21 days post-exposure to MWCNT. BALF was collected for total and differential cell counts (macrophages, neutrophils, eosinophils, lymphocytes) and for measurements of total protein concentration and lactate dehydrogenase (LDH) activity levels. We present herein the percentages of neutrophils, as it is not possible to compare effects of ENMs across laboratories or species using absolute neutrophil numbers due to variability related to lung size and lavage technique. The left lung was used for histopathological analyses. The details of lung delivery of ENMs to mice or rats, necropsy, tissue collection, and histopathology are described further (See Supplemental Materials, Supplemental Methods and Materials). All animals were treated humanely and with regard for alleviation of suffering.

Statistical analysis: Data are presented in the Figures as mean ± standard error of the mean for groups of 4 to 6 mice or rats. Two-way analyses of variance (ANOVA) and post-hoc Tukey's test or post-hoc Bonferroni t-test were performed. The analyses considered the main effects of and interactions between the factors, dose, and laboratory. In many cases, the two-way interaction was not statistically significant, so independent one-way analyses were performed. $P \leq 0.05$ was used to determine significant differences (Graphpad Prism 5, Graphpad Software, LaJolla, CA; SigmaPlot 11, Systat Software, Inc., San Jose, CA).

RESULTS

Mouse Working Group

***TiO_2*:** Differential counting of cells retrieved in BALF showed that all TiO_2 ENMs tested increased the percentage of neutrophils relative to other cell types (macrophages, lymphocytes, eosinophils). The percentages of neutrophils were compared as absolute numbers of neutrophils and other BALF cells were highly variable among the four different laboratories. Only results for the highest dose (40 μg/50 μl) are shown as lower doses had no significant effect on lung inflammation. TiO_2-P25 produced a significant increase in the relative percentages of neutrophils in two out of four laboratories at 1 day post-exposure (Figure 1A). TiO_2-A caused a significant increase in one out of four laboratories (Figure 1B), whereas TiO_2-NB caused a significant increase in neutrophils in three out of four labs (Figure 1C). Neutrophilic inflammation in response to all TiO_2 nanoparticles returned to nearly baseline levels by day 7 post-exposure. Macrophages comprised >95% of total BALF cells retrieved from the lungs of mice exposed to DM alone (data not shown). TiO_2 nanoparticles did not cause significant increases in the relative percentages of lymphocytes or eosinophils, with the exception of a slight increase in the relative percentage of eosinophils observed by one laboratory after treatment with TiO_2-NB (data not shown). BALF cytospins confirmed that inflammation caused by nanoparticle exposure was due primarily to neutrophil influx (data not shown). TiO_2-NB caused an inflammatory response at the terminal bronchiolar region and alveolar duct bifurcation region of the distal lung in mice 1 day after OPA delivery that was observed among four laboratories (Figure 2). TiO_2-NB were easily detectable by polarized light microscopy and were localized primarily in alveolar macrophages.

MWCNTs: Differential cell counting showed that at 1 day post-exposure all forms of MWCNT (O-MWCNT, P-MWCNT, and F-MWCNT) at 40 μg/50 μl increased the percentage of neutrophils in BALF of mice relative to other cell types (Figure 3). Lower doses of MWCNT caused no significant effects on neutrophilia. In mice treated with MWCNT, there were no significant increases in the relative percentages of lymphocytes or eosinophils (data not shown). Light microscopic images of BAL cytospins confirmed that changes in the relative percentage of BAL cells were due to a significant increase in neutrophils produced by treatment with MWCNT (data not shown). O-MWCNT caused the greatest increases in neutrophils at 1 day post-exposure in three out of four laboratories as compared to P- and F-MWCNT (Figure 3). The inflammatory responses to all MWCNTs subsided to control levels by 21 days post-exposure. Combining the data from four laboratories demonstrated the following order of potency: O-MWCNT > P-MWCNT > F-MWCNT, albeit not to a significant extent (see Supplemental Material, Figure S1). However, combining the data from three out of four laboratories (ML1, ML2, and ML3) that initially showed significant effects of MWCNTs on PMNs demonstrated a significant difference between O-MWCNT and F-MWCNT (Supplemental Material, Figure S1). All four laboratories demonstrated that total protein and LDH levels in BALF were increased by all forms of MWCNT or TiO_2 nanoparticles, although these two endpoints of lung injury were highly variable among laboratories (data not shown).

Histopathological analysis was used to determine the site of deposition for MWCNTs and the type of inflammatory lesions caused by oropharyngeal exposure to MWCNTs. Figure 4A shows that O-MWCNT delivered by OPA to mice caused centriacinar bronchiolitis/alveolitis in three out of four laboratories (ML1, ML2, and ML3). One laboratory (ML4) observed O-MWCNT primarily within alveolar ducts with little inflammation. There appeared to be far less O-

MWCNT delivered to the lungs by ML4 (Figure 4A), which correlated with much lower percentages of neutrophils recovered from mice in ML4 (Figure 3A). The inflammatory response associated with MWCNT exposure was characterized by neutrophilia as determined by BALF cell differentials (Figure 4B) and by using immunohistochemical staining with an anti-neutrophil antibody, which revealed neutrophils near terminal bronchioles and in proximity to macrophages containing O-MWCNT (Figure 4C).

Rat Working Group

TiO_2: Neither the spherical TiO2 nanoparticles (TiO_2-P25, TiO_2-A) caused dose-related changes in the percentage of BALF neutrophils in rats in any of the three laboratories, whereas TiO_2 nanobelts caused significant neutrophilia in a dose-dependent manner in two out of three laboratories (Figure 5A-C). The percentage and not the number of neutrophils is shown because cell number data was variable among the three laboratories, likely due to differences in lavage technique. However, there were visible TiO_2 inclusions in macrophages recovered in BALF from exposed rats, indicating that TiO_2 nanospheres reached the distal lung after IT. By day 7, all responses had returned to control values. There were no significant differences between control and TiO_2 exposed animals with respect to total protein and LDH assays (data not shown).

MWCNTs: Neutrophils were significantly elevated in rats exposed to the highest dose of all types of MWCNTs (200 μg/rat) compared to controls that received DM alone (Figure 6). A significant effect of dose was found by all laboratories (RL1, RL2, and RL3) such that the mid and high dose groups showed elevations in the percentage of neutrophils in comparison to controls at 1 day post-exposure. Histological evaluation of lung tissue sections demonstrated the presence of inflammatory cells within centriacinar regions similar to that observed in the mouse

working group (data not shown). Two out of three laboratories also observed a significant effect at the highest dose of O-MWCNT at 21 days post-exposure (data not shown). A significant effect of the highest dose of P-MWCNT was found in all laboratories at 1 day post-exposure, but reduced neutrophilia compared to O-MWCNT. A small but significant effect was observed by 2 out of 3 laboratories in the high dose P-MWCNT group at 21 days post-exposure. The percentage of neutrophils in BALF of rats exposed to F-MWCNT was significantly elevated at the highest dose in all laboratories at day 1, but the neutrophilia was reduced in comparison to O-MWCNT at 1 day and to O-MWCNT and P-MWCNT at 21 days post-exposure. Interlaboratory precision was high enough to combine results from labs. This permitted a MWCNT toxicity ranking (See Supplemental Material, Figure S2), which showed that O- and F-MWCNT caused stronger neutrophilic influx than the P-MWCNT at the 200 μg dose at 1 day post-exposure. At 21 days, there was no significant difference between rats that received DM or the highest dose of F-MWCNT. However, significant neutrophilia persisted at 21 days for rats exposed to O-MWCNTs and P-MWCNTs at the highest dose. There were no significant differences between control and MWCNT-exposed rats with respect to total protein and LDH assays (data not shown). Histopathological examination of the lungs demonstrated acute inflammatory lesions 1 day post-exposure, but not lasting changes 21 days post-instillation (data not shown).

DISCUSSION

The results presented herein comprise the first attempt by an integrated consortium of independent laboratories to determine whether the effects of well-characterized ENMs on pulmonary responses in mice and rats could be reliably reproduced by multiple investigators using a harmonized protocol. ENMs presented a challenge for inter-laboratory comparisons

given the complexity of variables, including dispersion and characterization. Despite variability between laboratories in endpoints such as cell counts, LDH and total protein in BALF, other endpoints revealed consistent patterns amongst laboratories and between rodent models, including neutrophilic inflammation (as a percentage of total cells), and pathologic responses in mice treated with TiO_2 nanobelts or O-MWCNTs. Because inhalation is a primary route of exposure to particles, the lungs represent a major target organ for the toxicity of ENMs from occupational, accidental, or environmental exposures (Kreyling 2010). High bolus doses of ENMs were used in this study and delivered by OPA in mice or IT in rats. The approach used in these studies was not intended to mimic real-world exposure conditions or deposition patterns in the lung that would occur via inhalation. Instead, we sought to evaluate whether different laboratories could generate similar and reproducible results with ENMs. Further work should focus on results following inhalation exposure, which is more physiologically relevant, to develop data for risk assessment and characterization to derive exposure standards.

The primary endpoint that was reliably reproducible in this inter-laboratory effort was acute neutrophilia. However, the four laboratories in the mouse group found different orders of potency for the three different types of TiO_2 nanoparticles. For example, ML1 showed that TiO_2-P25 produced the greatest level of neutrophilia at day 1, while ML3 showed that TiO_2-NB caused the highest level of neutrophilia. TiO_2 nanobelts were generally more toxic than TiO_2 nanospheres, perhaps due to their shape, and produced neutrophilia in nearly all laboratories. However, neutrophilia caused by TiO_2 nanoparticles did not persist through 7 days post-exposure. Therefore, although acute inflammatory endpoints may not be the most useful for determining chronic disease such as fibrosis or carcinogenesis, it is still the most sensitive endpoint for toxicity ranking. Future studies comparing spherical to high-aspect ratio TiO_2

should address clearance versus retention in the lungs after inhalation, evaluate pathologic changes over a period of weeks to months, and measure appropriate biomarkers of fibroproliferative and neoplastic disease.

All laboratories showed similar acute inflammatory responses to MWCNTs in mice and rats as indicated by significant neutrophilic influx into the lungs of exposed animals and similar lung pathology. Three out of four laboratories in the mouse group showed that O-MWCNT were the most inflammatory, as indicated by neutrophil influx at 1 day post-exposure, while F-MWCNT were the least pro-inflammatory. All three laboratories in the rat group also showed that O-MWCNT was the most inflammatory (Supplemental Material, Figure S2). This judgment about relative toxicological potency is based on the consistent findings of an onset of inflammation at a lower dose (i.e., 50 μg instilled) combined with persistence of neutrophilia at 21 days post-exposure. Therefore, both rodent groups in the Consortium effort independently identified O-MWCNTs as having the greatest proinflammatory effect, suggesting that residual catalytic metals (see Xia et al. (2013) for physicochemical characterization) may have contributed to the inflammatory response. Moreover, the mouse and rat working groups observed similar pathology after exposure to O-MWCNT.

In retrospect, we can identify aspects of the study design that worked well and those that could be considered for improvement in future inter-laboratory *in vivo* comparison studies with ENMs. The concordance in findings in terms of neutrophilia and histopathological changes that were detected with the anatase nanobelts and MWCNTs speaks to the careful planning and lends strength to conclusions made about the relative acute potency of these materials. The variability that was found, though, in the absolute number of neutrophils highlights some methodological differences that were not considered at the outset of the studies. Even though the rat and mouse

groups used similar dispersion protocols by design to break up large agglomerates, it was beyond the scope of this project to ensure that all users had identical dispersion instrumentation. Thus, it was not technically possible to ensure that all laboratories delivered ENMs that were identical in particle size distribution. Approaches that have been recently described (Taurozzi et al. 2011, 2012) could be considered for future efforts, but were not available at the time the studies were conducted.

Other difficult design problems to overcome include instillation/aspiration and lavage technique differences from one laboratory to another. Despite detailed planning, it was not realistic to mandate identical techniques across the laboratories because that would have involved significant expenditure of time for training and of animals. It may not be possible to overcome these problems and, so, careful consideration should be given to those endpoints that can be reliably used for comparisons. Lastly, inherently low-toxicity ENMs such as TiO_2 is perhaps not as useful for inter-laboratory comparisons as more potent materials. More specifically, the spherical TiO_2 particles were not potent enough in these bioassays to be identified consistently across the laboratories as having acute in vivo toxicity upon bolus delivery. The MWCNTs, on the other hand, were sufficiently potent to be identified as potentially hazardous by all laboratories in this round-robin effort, i.e., sufficiently insensitive to the methodological differences amongst the laboratories.

It is important to note that pathology might be impacted by nanoparticle delivery modes. The fibrotic response to MWCNT in mice has been reported to be more diffuse in inhalation exposures as compared to aspiration (Shvedova et al. 2008). The issue of MWCNT dispersion (i.e., agglomeration state) and consequent disease is a critical one in assessing health risks. For example, inhalation of agglomerated MWCNT causes a different pathology (less interstitial

fibrosis) than dispersed fibrillar structures (Ma Hock et al. 2009; Pauluhn, 2010). Other recent findings using OPA aspiration of well-dispersed MWCNT caused more fibrosis and growth factor production than non-dispersed MWCNT (Porter et al. 2010; *Wang et al. 2011*). All laboratories in the Consortium used the same dispersion medium for suspending MWCNT or TiO_2 nanoparticles. Nevertheless, it was apparent that some agglomeration occurred, particularly for MWCNTs. Future studies should carefully evaluate whether functionalization of MWCNTs or other ENMs influences agglomeration status and surface properties, which could in turn alter pathologic responses upon bolus delivery to the lungs in a liquid suspension. Responses following more realistic and physiological inhalation exposures should be evaluated with these materials in order to confirm the hazard ranking and to derive the lowest or no-effect levels for purposes of risk characterization which, however, was not the purpose of this paper.

MWCNTs and TiO_2 nanobelts represent ENMs that are referred to as high-aspect ratio nanoparticles, i.e., fiber-like structures that have nanoscale width but can be micrometers in length. A principal characteristic of high-aspect ratio nanoparticles that is shared by pathogenic fibers such as asbestos is impeded clearance from the lungs after inhalation exposure, leading to the pathogenesis of diseases such as pulmonary fibrosis and mesothelioma (Bonner 2010). There is evidence that high-aspect ratio TiO_2 nanobelts and long MWCNTs are more pathogenic and may elicit "frustrated phagocytosis" by macrophages, lysosomal disruption and impaired clearance (Hamilton et al. 2009; Donaldson et al. 2011). Therefore, some of the ENMs used in this Consortium study represent those that could create concerns about human health if exposures were to occur.

Pulmonary toxicology studies in rodents have shown that OPA or IT exposure to MWCNTs at high doses/concentrations, like asbestos fibers, results in lung inflammation and interstitial

pulmonary fibrosis (Han et al. 2010; Mercer et al. 2011; Porter et al., 2010; Ryman-Rasmussen et al. 2009a; Wang et al. 2011). Inhaled MWCNTs can also migrate to the pleural membrane surrounding the lungs to cause inflammatory reactions (Porter et al. 2010; Ryman-Rasmussen et al 2009b). Other MWCNTs have also been reported to induce mesothelioma in genetically susceptible mice that lack an allele of the tumor suppressor p53 (Takagi et al. 2008) after direct intraperitoneal injection of very high doses. Some caution should be taken in interpreting these latter results as p53-deficient mice spontaneously develop tumors. In addition, a two year study showed that MWCNT possessed no carcinogenicity when injected into the peritoneal cavity of male Wistar rats (Muller et al. 2009). It is noteworthy, though, that this study used very short MWCNT (<1 μm). However, these injection studies did not address direct exposure to the lungs. It should also be stressed that it is unclear to what extent the differences in MWCNT properties (i.e., associated metal content, dispersion state/method, aspect ratio, rigidity) and means of exposure played a role in disparate outcomes. Therefore, whether or not MWCNTs are carcinogenic in inhalation studies remains to be determined.

So far, no human disease of any kind has yet to be linked to exposure to carbon nanotubes. Therefore, the scientific community has a unique opportunity to address human health effects in a preventive manner and reduce potential adverse health effects by establishing assays to predict disease outcome using *in vitro* and *in vivo* models. The present article on inter-laboratory studies in rodents exposed to ENMs and the accompanying article in this issue on inter-laboratory *in vitro* studies comprise one of the first attempts to assess the reproducibility of experiments among different laboratories using harmonized experimental protocols and identical ENMs.

OUTLOOK AND FUTURE DIRECTIONS

A vast array of industrial and consumer products are emerging on the market as part of the nanotechnology revolution. A substantial number are already present in the marketplace in consumer products and a comprehensive list is maintained and updated by the Project on Emerging Technologies at the Woodrow Wilson International Center for Scholars (www.nanotechproject.org). It is not feasible or practical to test all of these ENMs using *in vivo* rodent models. Predictive high-throughput screening of materials using validated *in vitro* model systems coupled with more thorough analyses of selected ENMs using realistic *in vivo* rodent models and realistic exposures will likely be the only practical way to determine the toxic potential of an enormous variety of emerging nanomaterials (Nel et al. 2009). It is anticipated that standardized protocols will be used for *in vitro* screening and the resulting data made publicly available through a centralized database. In addition, a centralized repository for ENMs should be established to provide well-characterized ENMs in large quantities to investigators.

We are only beginning to understand the mechanisms of toxicity for the increasing variety of emerging ENMs. Growing evidence indicates that nanosizing particles increases toxicity because of a proportional increase in surface area that is then available to generate ROS, a primary factor that drives cellular stress and disease pathogenesis (Nel et al. 2006). However, in addition to the obvious role of increased surface area and ROS generation, nanosized particles could be capable of moving across cellular barriers to interact with subcellular structures (e.g, mitochondria, microtubules, organelles, DNA) in potentially unique ways that we have yet to fully comprehend. There is also evidence that ENMs traverse the pulmonary epithelial-endothelial barrier to gain access to secondary target tissues (Aiso et al. 2011; Kreyling et al. 2002, 2009). Alternatively, ENMs could influence immune responses of secondary target tissues by

stimulating the release of lung cytokines into the systemic circulation (Mitchell et al. 2007). Therefore more research to elucidate common mechanisms of nanoparticle-induced adverse health effects is required to keep pace with the emerging nanotechnology industry and associated human and environmental exposures.

Unfortunately, funding for nanotoxicology research on health effects lags far behind the amount of funding that is available for nanotechnology research and development. The disproportionate emphasis on nanotechnology research and development relative to issues of risk for human health and the environment could result in a new wave of occupational and environmental health crises. While many ENMs will present little or no risk, it is inevitable that at least some ENMs will pose a significant risk to human health and the environment. Our Consortium experiments described herein are therefore significant in providing results of a coordinated effort towards addressing hazard identification of high priority ENMs. Future research should expand this consortium effort to allow for toxicity testing and exposure assessment to ensure the safe continuation and economic viability of nanotechnology.

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FIGURE LEGENDS

Figure 1. Stimulation of neutrophilic inflammation in the lungs of mice by TiO_2 ENMs. A) Percentages of neutrophils (PMNs) in BALF among four laboratories 1 day after OPA exposure to rutile/anatase P25 nanospheres (TiO_2-P25), B) anatase nanospheres (TiO_2-A), or C) anatase nanobelts (TiO_2-NB). *P<0.05, **P<0.01, or ***P<0.001 compared to DM-exposed controls.

Figure 2. Histopathology showing inflammation at the terminal bronchiolar/alveolar duct bifurcation region 1 day after OPA exposure to TiO_2 nanobelts (TiO_2-NB) in C57BL/6 mice from four laboratories: A-C: ML1, D-F: ML2, G-I: ML3, J-L: ML4. Left hand panels (A, D, G, and J) are bright field light microscopy of representative sections of lung from mice that received dispersion medium (DM) alone as a vehicle control. Middle panels (B, E, H, and K) represent lung sections from mice that received TiO_2-NB (40 μg/50 uL) suspended in DM showing inflammatory lesions primarily localized to alveolar duct bifurcations. Right hand panels (C, F, I, and L) show polarized light microscopy of the same images in B, E, H, and K showing inflammation at an alveolar duct bifurcation caused by TiO_2-NB 1 day after OPA. TiO_2-NB were found within macrophages at alveolar duct bifurcations as indicated by arrow. The alveolar ducts (ad), terminal bronchioles (tb), blood vessel (bv), alveolus (a), and airway epithelium (e) are indicated. Lung tissues were stained with hematoxylin and eosin and photomicrographs taken at the same magnification. Inflammatory foci are indicated by asterisks. Scale bar, 50 μm.

Figure 3. Stimulation of neutrophilic inflammation in the lungs of mice by O-MWCNT, P-MWCNT, and F-MWCNT. A) Four laboratories (ML1, ML2, ML3, ML4) showed that O-MWCNT delivered at a dose of 40 ug/50uL by OPA caused a significant increase the percentage

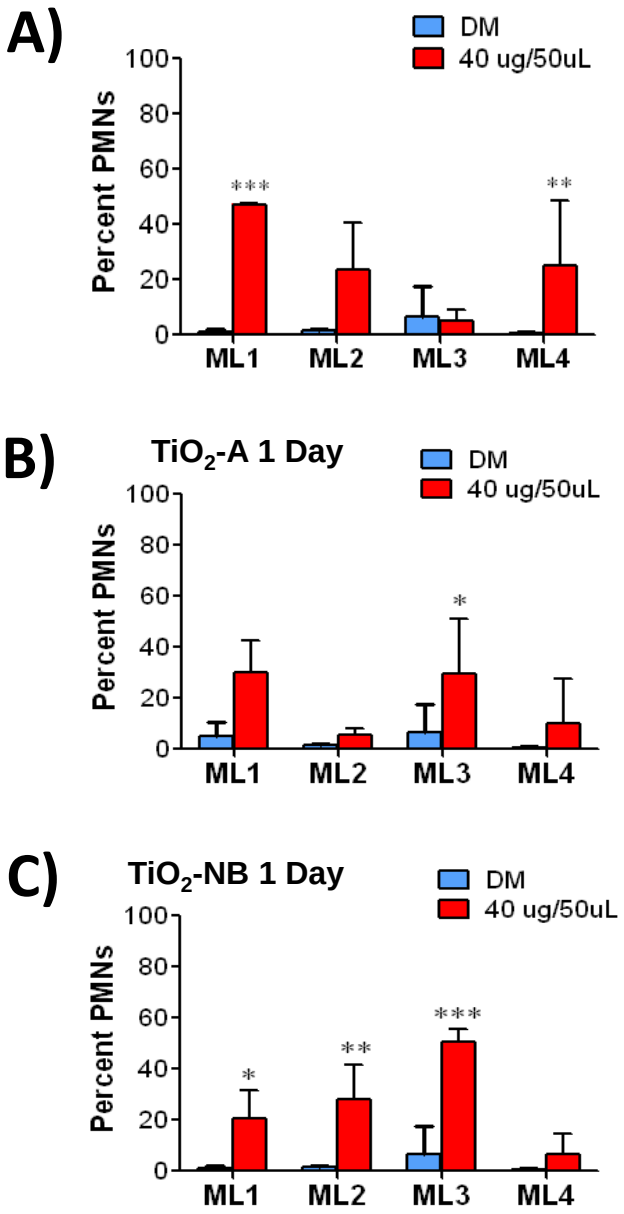
of neutrophils (PMNs) at 1 day post-exposure in BAL fluid of mice. **P<0.01, or ***P<0.001 compared to DM. B) Effect of P-MWCNT towards increasing PMNs at 1 day **P<0.01 compared to DM. C) Three laboratories show a significant effect of F-MWCNT on increasing PMNs, albeit to a lesser extent compared with O-MWCNT. *P<0.05 compared to DM.

Figure 4. A) Histopathology showing lung inflammatory response to O-MWCNTs. Centriacinar bronchiolitis/alveolitis (indicated by dashed arrows) was induced by O-MWCNT (40 μg/50 μl) in three out of four laboratories (ML1, ML2, ML3). A fourth laboratory (ML4) showed some deposition of O-MWCNT in alveolar ducts with marginal inflammation. Macrophages containing O-MWCNT are indicated by solid arrows. B). BAL cytospin images of cells retrieved from the lungs of mice 1 day after exposure to DM by OPA showing >95% macrophages or after exposure to O-MWCNT (40 μg/50 μl) by OPA showed enlarged, activated alveolar macrophages with numerous MWCNT inclusions (solid arrows) and neutrophils that do not contain MWCNT (dashed arrows). The images are from a single laboratory (ML3) but are typical of responses from the ML1 and ML2 laboratories. C) Immunohistochemistry using a monoclonal rat anti-mouse neutrophil (allotypic marker clone 7/4) antibody showing location of neutrophils (dashed arrows) near terminal bronchioles and in relation to macrophages containing O-MWCNT (solid arrows). Representative data are from ML3.

Figure 5. Stimulation of neutrophilic inflammation in the lungs of rats by TiO_2 ENMs. A) Percentages of neutrophils (PMNs) in BALF among two laboratories 1 day after IT exposure of rutile/anatase P25 nanospheres (TiO_2-P25), or B) anatase nanospheres (TiO_2-A). C) Percentages of PMNs in BAL fluid among three laboratories 1 day after exposure to anatase nanobelts (TiO_2-NB). *P<0.05 compared to DM-exposed controls.

Figure 6. Stimulation of neutrophilic inflammation in the lungs of rats by MWCNTs as demonstrated in three laboratories (RL1, RL2, RL3). Percentage of neutrophils in the BALF of rats 1 day after treatment with A) O-MWCNT, B) P-MWCNT, or C) F-MWCNT. *$P < 0.05$ as compared to DM-exposed controls.

Fig. 1


A)
DM
40 ug/50uL
Percent PMNs
100
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60
40
20
0
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ML1
ML2
ML3
ML4
B)
TiO2-A 1 Day
DM
40 ug/50uL
Percent PMNs
100
80
60
40
20
0
*
ML1
ML2
ML3
ML4
C)
TiO2-NB 1 Day
DM
40 ug/50uL
Percent PMNs
100
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ML1
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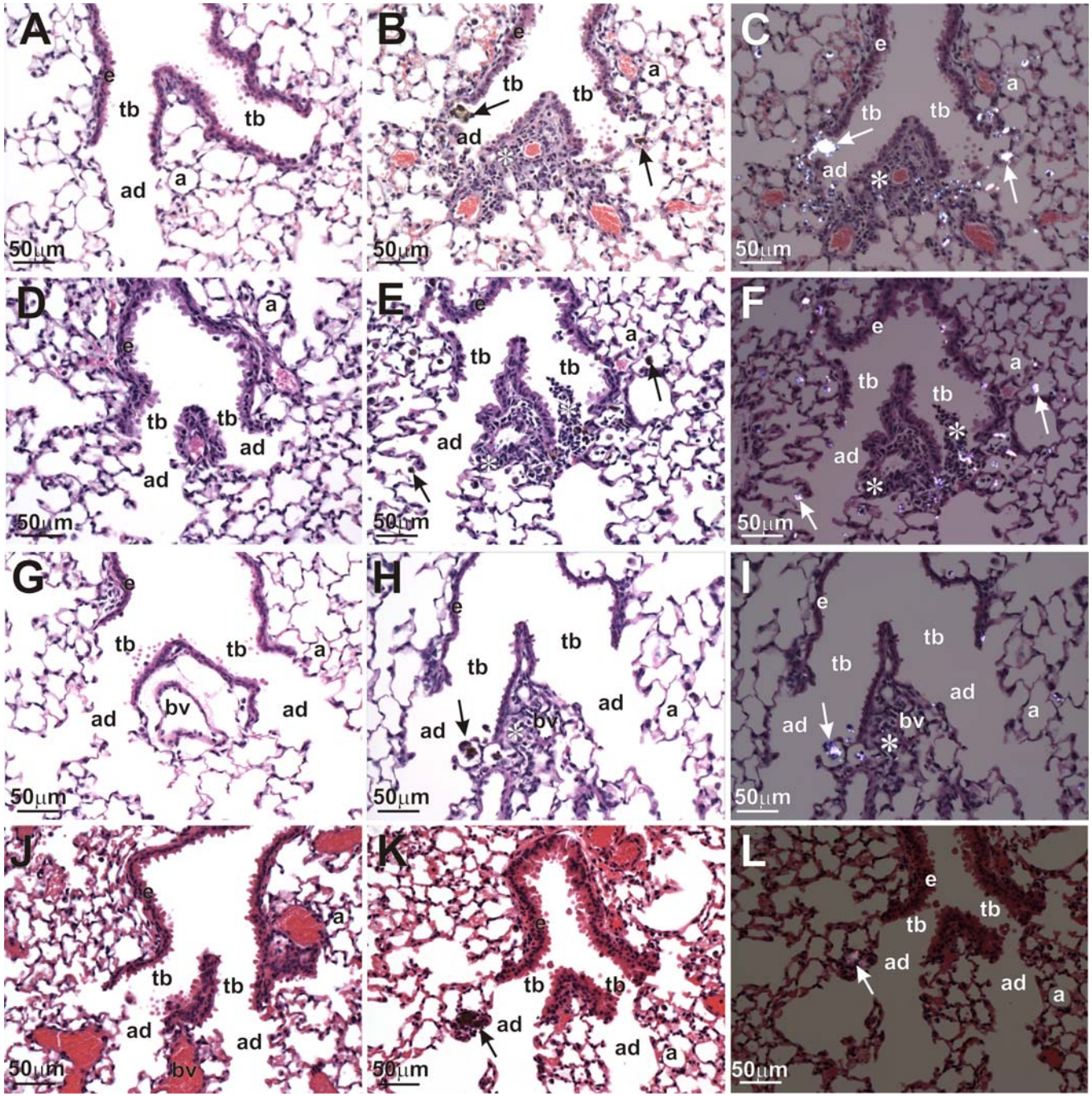
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Fig. 2

Fig. 3

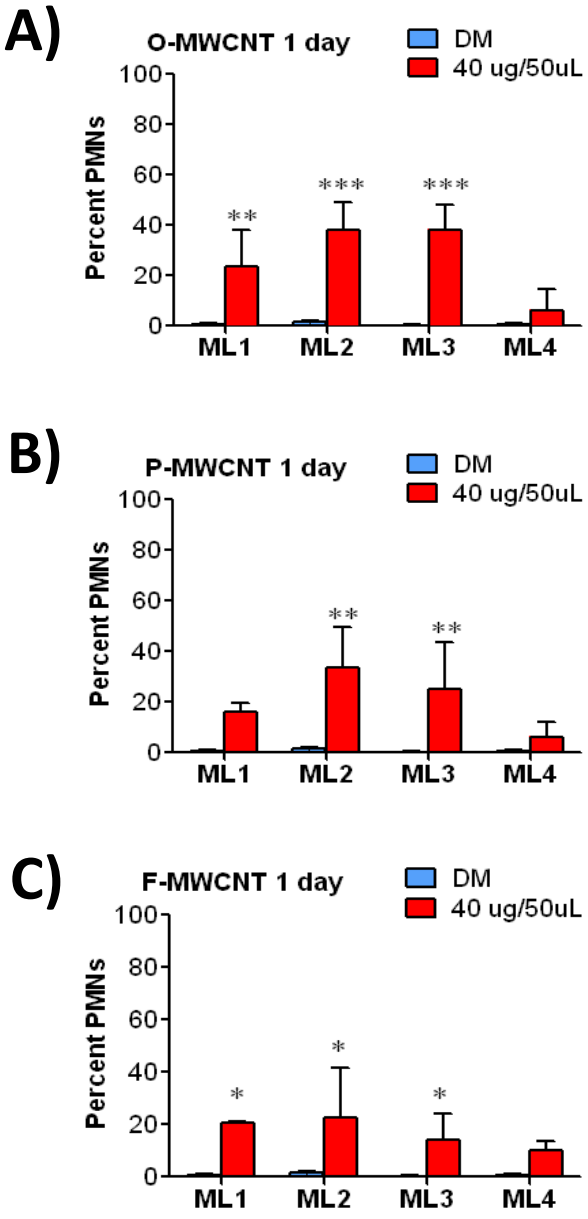

A)
O-MWCNT 1 day
DM
40 ug/50uL
Percent PMNs
100
80
60
40
20
0
**
***
***
ML1
ML2
ML3
ML4
B)
P-MWCNT 1 day
DM
40 ug/50uL
Percent PMNs
100
80
60
40
20
0
**
**
ML1
ML2
ML3
ML4
C)
F-MWCNT 1 day
DM
40 ug/50uL
Percent PMNs
100
80
60
40
20
0
*
*
*
ML1
ML2
ML3
ML4

Fig. 4

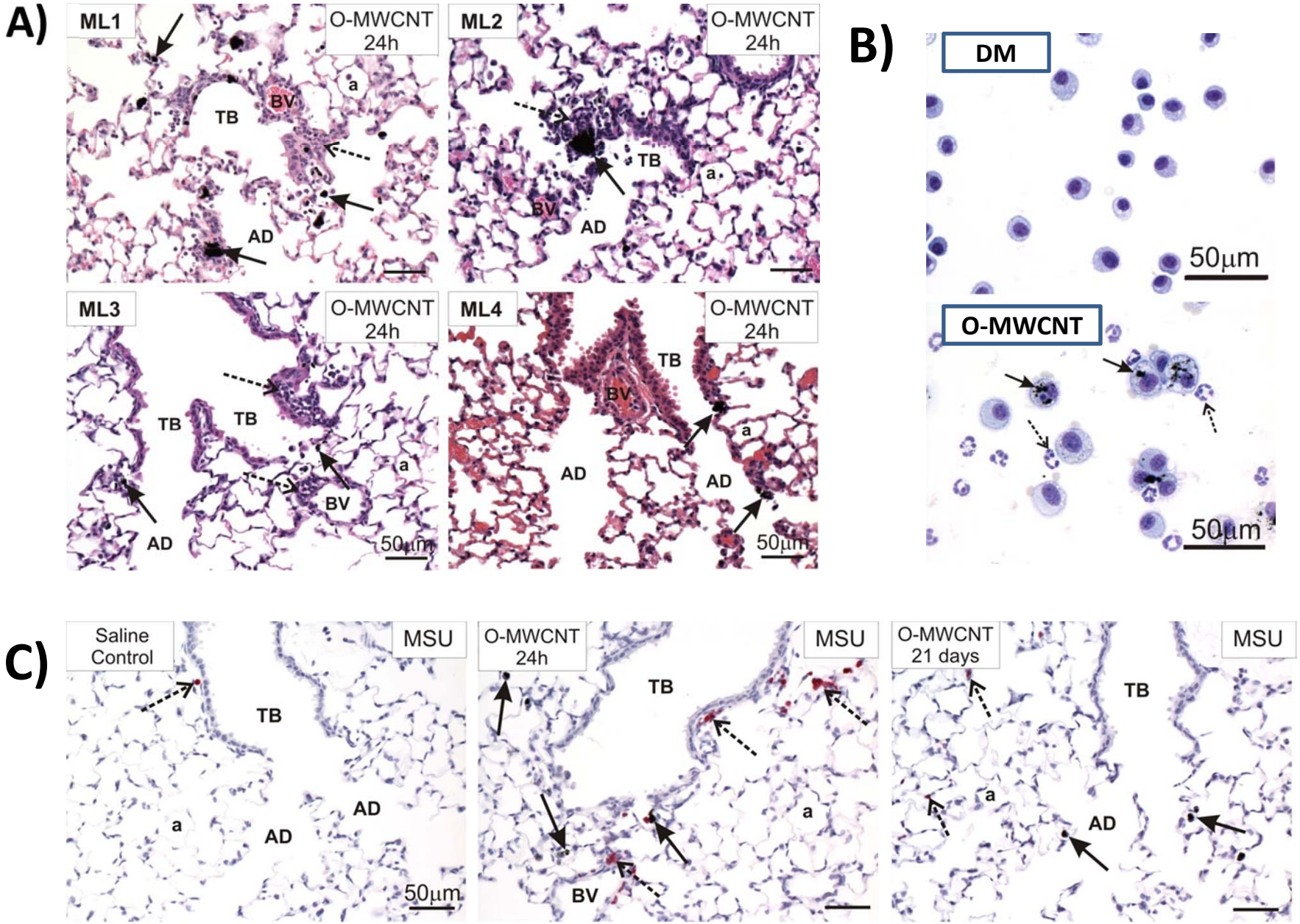

A)
ML1
O-MWCNT 24h
TB
BV
a
AD
ML2
O-MWCNT 24h
TB
a
BV
AD
ML3
O-MWCNT 24h
TB
TB
a
BV
AD
50μm
ML4
O-MWCNT 24h
TB
BV
a
AD
AD
50μm
B)
DM
50μm
O-MWCNT
50μm
C)
Saline Control
MSU
TB
AD
a
AD
50μm
O-MWCNT 24h
MSU
TB
a
BV
O-MWCNT 21 days
MSU
TB
a
AD

Fig. 5

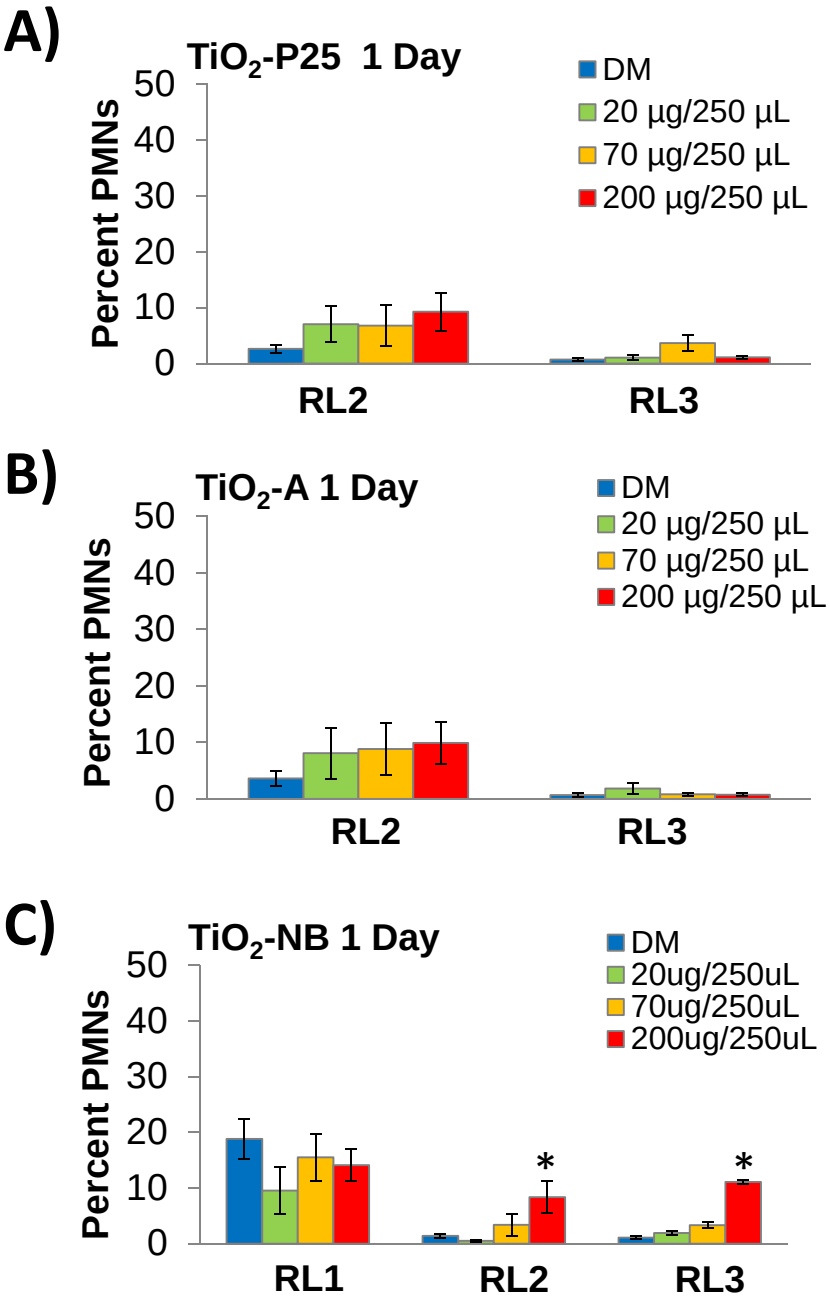
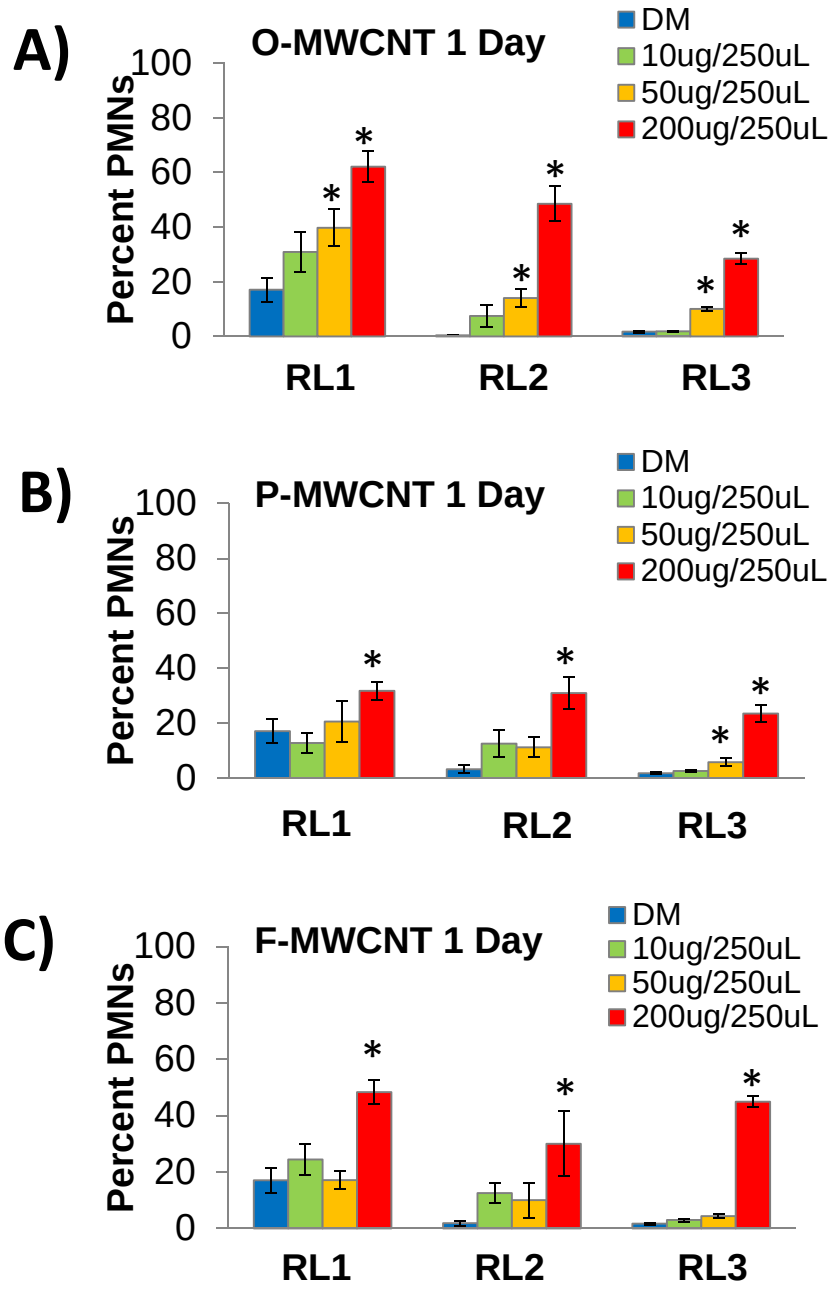

A)
TiO2-P25 1 Day
DM
20 µg/250 µL
70 µg/250 µL
200 µg/250 µL
Percent PMNs
50
40
30
20
10
0
RL2
RL3
B)
TiO2-A 1 Day
DM
20 µg/250 µL
70 µg/250 µL
200 µg/250 µL
Percent PMNs
50
40
30
20
10
0
RL2
RL3
C)
TiO2-NB 1 Day
DM
20ug/250uL
70ug/250uL
200ug/250uL
Percent PMNs
50
40
30
20
10
0
*
*
RL1
RL2
RL3

Fig. 6


A)
O-MWCNT 1 Day
DM
10ug/250uL
50ug/250uL
200ug/250uL
Percent PMNs
100
80
60
40
20
0
RL1
RL2
RL3
B)
P-MWCNT 1 Day
C)
F-MWCNT 1 Day